MORPHOLOGICAL AND BIOCHEMICAL ASPECTS IN STRAWBERRY LEAVES UNDER THE INFLUENCE OF BIOLOGICAL CONTROL AGENTS

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Abstract: *The purpose of this study was to reveal by optical microscopy and ultra-microscopy the changes induced in the cells and tissues of strawberry plants by different treatments with elicitors obtained from fungal strains to activate various metabolic pathways involved in the defense response of plants against pathogens. The diseases control is generally based on use of chemical pesticides, genetic resistant plant species and adequate agro-technical measures. Environment pollution with pesticide residues and the long period necessary to obtain genetic resistance in crop plants led to finding strategies based on inducing plant defense mechanism by appropriate stimuli. Thus, research has demonstrated the possibility to induce plant resistance to various pathogens from genera Fusarium, Plasmopara, Verticillium, Erwinia, Pseudomonas. Attempts have been done to assess the morphological and biochemical basis of resistance in plants of tomato, sun-flower, grapes or cotton. In vitro and in vivo experiments have been carried out in order to study and reveal the modifications in strawberry leaf blade tissues and cells ultra-structure during the defense response to pathogen Botrytis cinerea induced by E1, E2 and E3 preparations of microbial origin. Leaves sampled from strawberry plants cv. Senga Sengana have been examined by optical and electron microscopy. Immunodiffusion tests have been carried out from plant extracts with biochemical chemical composition.The comparative effect of pathogen control measures was assessed by monitoring plant health paramenters. The phenomenon of induced plant resistance was approached by new and modern interdisciplinary methods, including connections between plant histology, pathology and immunology .In vitro and in vivo experiments brought evidence that practical use of fungal preparations as signals to induce plant resistance to pathogen Botrytis cinerea, has a scientific base, improving defense mechanisms. The paper present images and results of measurements carried out in leaf blade tissues and evidences of thickening of mesophyllum and epidermis. Intracellular accumulations of phenols were evidenced proving the activation of defense mechanisms. The paper presents original data and photographs in a new scientific field represented by plant immunology and use of natural mechanisms for increasing strawberry resistance to pathogen Botrytis cinerea.*
Research was supported by PNCDI II Research Program, Projects nos. 31-078/2007 and 52-112/2008.

***Key words*:** *plant resistance, fungal extract, strawberry, Botrytis cinerea, leaves morphology*

INTRODUCTION

Plants have developed a variety of mechanisms of resistance to pathogen colonization and parasitic agents. KOMBRINK and SOMMSSICH (1995) classify the plant response against pathogen attack by spatio-temporal criteria in three broad categories: a) early response immediately after pathogen attack, b) local gene activation in tissues in close proximity with the infected site, c) systemic gene activation.

Many of these reactions have been extensively studied both in cell cultures and in

plants infected with pathogens, leading to the conclusion that the mechanisms are essentially the same in the simplified system interaction or real host - pathogen (HAHLBROCK et al., 1995, SOMSSICH and HAHLBROCK, 1998). Initial response of cells invaded by pathogen or treated with elicitor occurs within minutes and is followed quickly by a local gene activation.

Disease is a major factor affecting production in strawberry (*Fragaria x Ananassa* Duch.). Strawberries cultivated in Europe are affected by numerous pathogens: *Phytophthora* spp., *Colletotrichun acutatum*, *Phytophthora fragariae* var. *fragariae*, *Verticillium dahliae*, *Botrytis cinerea*, *Sphaerotheca macularis*, *Rhizopus nigricans* (MAAS, 1998).

Resorting to chemical treatments to control these diseases is expensive, have a negative impact on the environment and lower market value of the treated fruit. An alternative strategy is to increase plant resistance to these pathogens, including among other methods elicitor treatments (LEGARD et al., 2002).

The purpose of this study is to assess by optical and electron-microscopy the changes induced at the cellular level by different treatments with elicitors obtained from fungal strains of *Trichoderma viride*, *Penicillium chrysogenum* and *Botrytis cinerea* in strawberry for intensifying various metabolic pathways involved in the response of plant defense against pathogens.

MATERIAL AND METHODS

Greenhouse experiments

Vegetal material: strawberry plants cultivar Senga Sengana grown in greenhouse and regenerats obtained by multiplying *in vitro*.

Leaves taken from plants grown in greenhouse were measured to estimate total foliar surface and then they were sectioned transversely at the central rib for the aspect of tissue and thickening of cellulose and lignin.

Strawberry culture includes the variants: control (no treatment), chemical treatment (Captan, Teldan, Batron, Topsin) and biocontrol agents E1, E2, E3, which induce the plant defense reactions.

Variants had 5 replicates each.

Laboratory experiments

Regenerants were obtained by the multiple axial multiplying on Murashige-Skoog nutrient medium composed of macro-and micro-elements, Gamborg B5 vitamins, indolilbutiric acid, giberelic acid, benzilaminopurina, sucrose and agar (CACHITA-COSMA, 1987).

In case of regenerants, experimental design was maintained excepting chemical treatment option that was replaced by distilled water treatment.

In both cases, four treatments were performed at an interval of 48 hours. In each experimental variant the dose of treatment was applied by spraying the leaves. At 24 hours after the last treatment samples were taken for microscopy. For electron microscopy observations of leaf tissue, fragments were processed according to standard techniques

to be viewed in a Tesla electron microscope. For microscopy, 1-2 mm sections were stained with toluidine blue in borax.

To study the immunological aspects of interactions between fungal elicitors and plant the radial immunodiffusion method was adapted.

Percentage of leaves affected, assessed after the treatments, was used as a parameter for assessing the efficiency of application and the responsiveness of strawberry plants grown in greenhouse or *in vitro* systems.

RESULTS AND DISCUSSIONS

Leaves taken from plants in greenhouse were measured to estimate leaves surface

(Table 1).

The anatomical measurements at leaves level was analyzed by aspect of tissue around the median rib and thickening of cellulose and lignin, highlighted by photographs (Table 2).

Table 1

Statistical analysis of the influence of antifungal treatments on total foliar surface of strawberry

Treatment – on plant				
Experimental variant	Relative values cm^2	Dif. cm^2	Dif. %	Semnif.
Control	586.00	0.00	100.00	Mt
Chemical treatment	418.00	-168.00	71.33	OOO
E1	621.00	35.00	105.97	**
E2	817.00	231.00	139.42	***
E3	715.00	129.00	122.01	***
DL5% = 15.810 DL1% = 26.170 DL01% = 48.990				
Semnif exp.	***			

Table 2

Results of biometric measurements made at leaves of strawberry cultivar Senga Sengana – greenhouse culture

Variant of treatment	Upper epidermis	Mesophyll	Lower epidermis
Control	31,00	151,00	18,00
Chemical treatment on plant	26,00	210,00	19,00
Biological control agent E1 on plant	31,00	168,00	18,00
Biological control agent E2 on plant	36,80	159,20	18,40
Biological control agent E3 on plant	34,40	173,60	19,20

Control variant leaves in greenhouse experiment show mezophyl of 151.00µ, upper epidermis of 26.00µ and lower epidermis of 18.00µ. The midrib vein contains both xylem and phloem, with 20 layers of xylem vessels accompanied by xylem parenchyma. Parenchymatic issue under the vascular bundle is split into an external area of tabular collenchyma consisting of 3 layers of cells with walls thickened with cellulose and 3-4 layers of cellulosic parenchyma (Fig.1). To plants with chemical treatment in the greenhouse, the mesophyll and lower epidermis have a higher value than the control, while the upper epidermis is smaller compared to the control variant. Mid rib comprises a bundle with 23 layers of xylem vessels, and another one that is smaller. The tabular collenchyma consists of two layers of cells (Fig. 2).

For E1 fungal extract applied to plants in the greenhouse mesophyll is higher than the control values, while the thickness of both epidermis is equal to control. The midrib shows a bundle consisting of 14 layers of vessels, tabular collenchyma consists of two layers of cells and cellulosic parenchyma of four layers (Fig. 3).

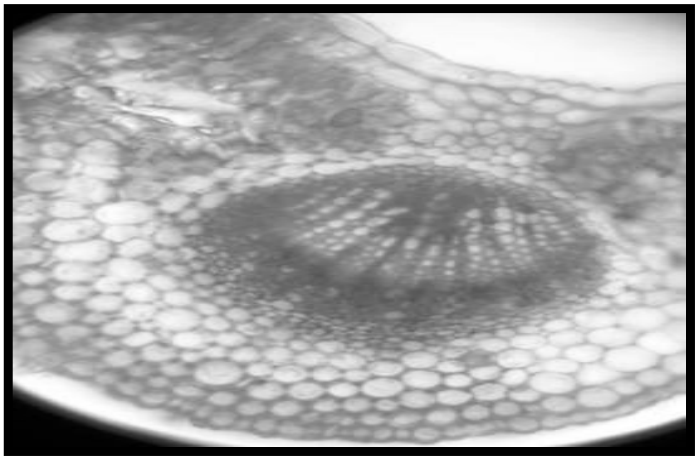

Figure 1: Aspect of strawberry leaf tissue - control

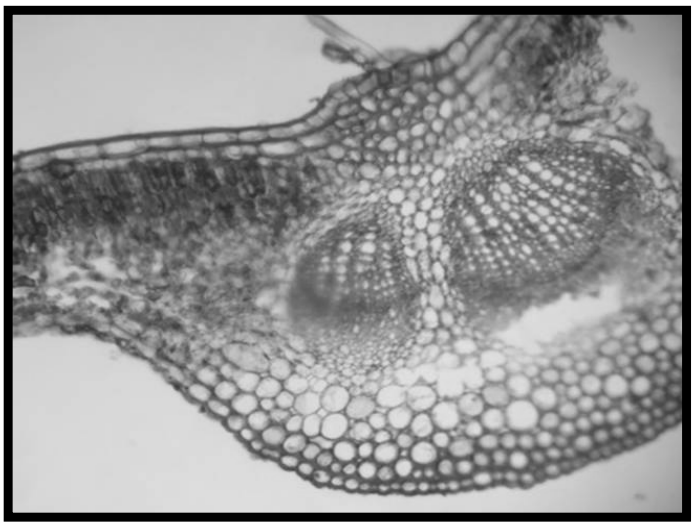

Figure 2: Aspect of strawberry leaf tissue – chemical treatment

Variants with control agents E2 and E3 show all values respectively mesophyll, upper epidermis, lower epidermis, higher than those of controls. Midrib is composed of a great vascular bundle with 17 layers of xylem vessels and cellulosic parenchyma is collenchymatic, stronger in the subepidermal layer.

Similar results were reported for tissues of soybean plants (*Glycine max* L.Merrill) line PR91M10, sensible to *Botrytis cinerea*, treated with the same fungal extracts to stimulate defense mechanisms (GEORGESCU et al., 2010).

In vitro, strawberry regenerants responded in a complex way to treatments with fungal

elicitors with gradual manifestation of some forms of stress. Growth and differentiation was slower in variants with fungal elicitors. Treatment with distilled water did not affect regenerants physiology.

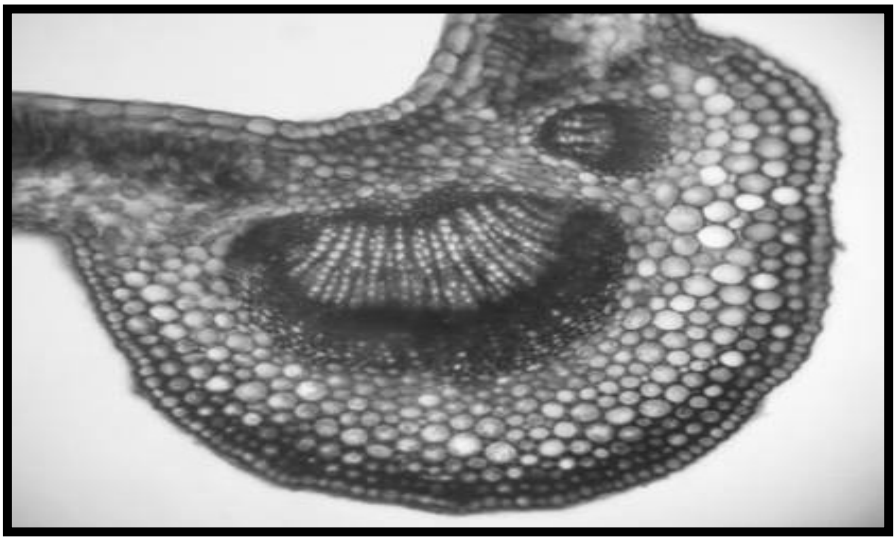

Figure 3: Aspect of strawberry leaf tissue treated with biocontrol agents

Analysis of leaf tissue structure and ultrastructure revealed the mosaic of responses to the action of fungal elicitors, modiffied cells with obvious phenomena of stress coexisting with normal-looking cells.

Many electronodens deposits precipitated on the tonoplast and plasmalema are possible breakdown or phenolic compounds. Similar results were reported by other authors for different plant species subject to the influences of various types of stress (salt, water, xenobiotic).

Withdrawal from the cell wall plasma membrane can be seen in Fig.4, but other authors have highlighted this phenomenon *in vitro* model systems (BOLWELL et al., 2001). The presence of phenolic compounds has been evidenced in the plastids, intercellular spaces, cell wall and tonoplast (Fig.4). This is consistent with other studies that emphasize the role of phenolic compounds and phytoalexins, to develop resistance in plants to different pathogens.

Interaction of plant antibodies with fungal elicitors from extracts assessed by radial immunodiffusion, is presented in Fig. 5, where the different appearance of circles of precipitation (as shape, thickness, color) of extracts containing elicitors is due to different molecular weight and specific concentrations as compared with the appearance of control solution, lacking elicitors.

The percentage of affected leaves in both experiments depended on the treatment applied on leaves. In compared with total number of healthy leaves from control strawberry plants the percentage of leaves attacked by pathogen was the lowest when the leaves of strawberry were treated with E2 biocontrol agent (Fig. 6)

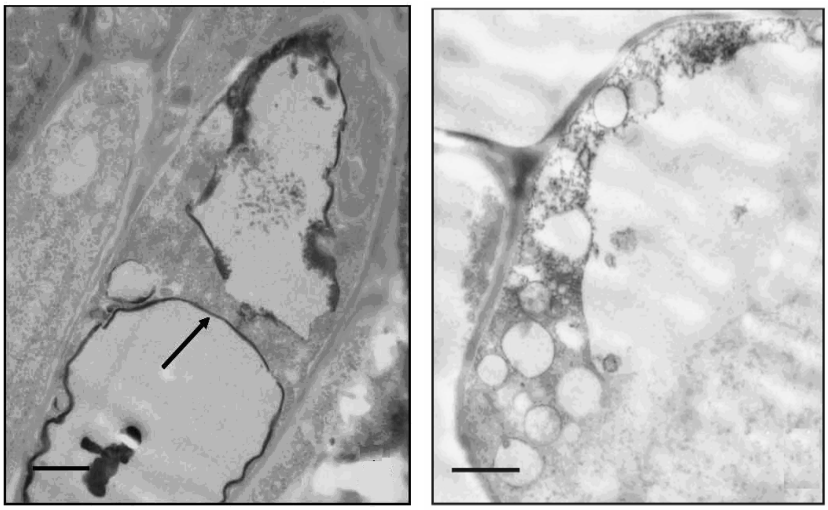

Figure 4: Effect of treatment with the fungal elicitors on the cells strawberry leaves:deposition of phenolic compounds in the cell wall, Bar: 1µm (left); Chloroplast after treatment with fungal elicitors: withdrawal of plasma membrane (pm): dark coloration is probably the result of plasmalem lipid peroxidation, Bar: 1µm (right).

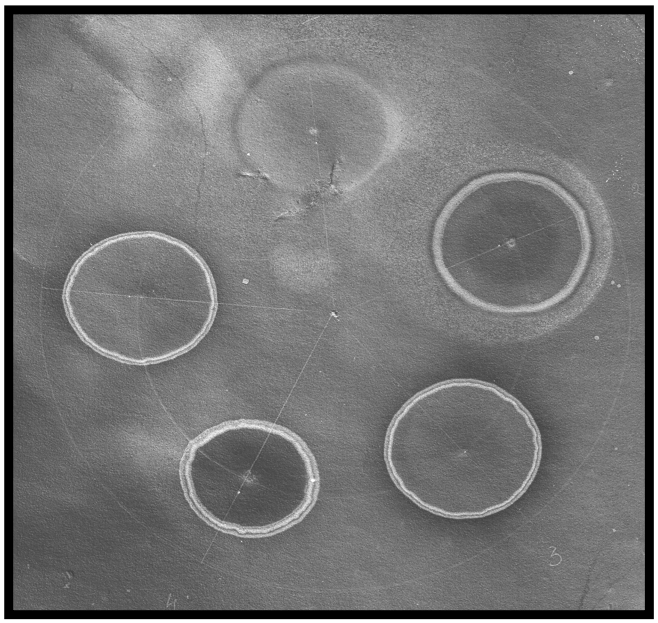

Figure 5: Interaction of strawberry antibodies with biocontrol agents from extracts assessed by radial immunodiffusion

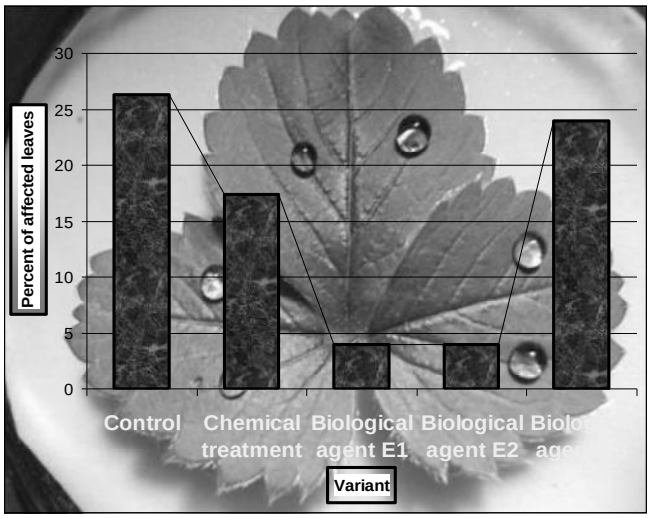

Figure 6: The influence of antifungal treatments on percent of affected leaves of strawberry plants cultivated in greenhouse - treatment on plant

CONCLUSIONS

The treatments with biological control agents E2-E3 on strawberry plants cultivated in the greenhouse determined the thickening of the mesophyll and of both epiderme, as compared with control

In leaves treated with fungal elicitors both cell organelles with the structure conforming with development stage and tissue specificity and cell organelles with altered structure as well as frequent alterations in the membrane and in the plastidial system were observed.

The deposits of the electrondense material suggest an intensification in synthesis and oxidation of the phenolic compounds, showing the typical defense reactions at plants.

The ultra-structural changes were not observed in control or in the variant treated with distilled water, thus attesting the induction of immune response at intracellular level due to elicitors from fungal extracts administered.

The strawberry plants from the variety Senga Sengana had different response reactions to biocontrol agents applied during the treatment.

The percentage of leaves attacked by pathogen was the lowest when the leaves of strawberry were treated with E2 biocontrol agent.

The research was supported by PNCDI II Research program, Projects nos. 31078/2007 and 52112/2008.

BIBLIOGRAPHY

1.BOLWELL, P., PAGE, A., PISLEWSKA, M., WOJTASZEK, P., 2001 Pathogenic infection and the oxidative defences in plant apoplast. Protoplasma, 217:20-32.

2.CACHITA-COSMA, D., 1987 Metode in vitro la plantele de cultura, Ed. Ceres, Bucuresti, 182-225.

3.GEORGESCU, M.I., SAVULESCU, E., LUCHIAN, V., MATEI, S., 2010 Dynamic blade components registered on soybean plants following treatments applied to soil and plant, in relation to resistance to attack of the fungus *Botrytis cinerea*, Sci. Papers, UASVM, Bucharest,

series A, vol.LIII, 504-508.
4.HALBROCK K., D. SCHEEL, E. LOGEMANN, T. NÜRNBERGER, M. PARNISKE, S. REINOLD, W.R. SACS, E. SCHMELZER, 1995 Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells, Proc.Natl.Acad.Sci.USA, 92, 4150-4157.
5.KOMBRINK E., I.E. SOMSSICH, 1995 Defence responses of plants to pathogens. In: J.H. Andrews, I.C. Tommerup, eds. Advances in Botanical Research, 21, 1-34.
6.SOMSSICH I.E., K. HAHLBROCK, 1998 Pathogen defense in plants - a paradigm of biological complexity, Trends Plant Sci., 3, 86-90.
7.LEGARD D.E., J.C. MERTELY, C.K. CHANDLER, J.F. PRICE, J.D. DUVAL, 2002, Management of strawberry diseases in the 21st century: chemical and cultural control of *Botrytis* fruit rot. In: S.C. Hokanson and Jamieson A.R.(eds) Strawberry Research to 2001: Proceeding 5th North American Strawberry Conference, ASHS Press, Alexandria, VA, 58-63.
8.MAAS J.L., 1998 Compendium of Strawberry Diseases, APS Press, St. Paul, MN, USA.